Alpha$_1$ Adrenergic Receptor–induced c-*fos* Gene Expression in Rat Aorta and Cultured Vascular Smooth Muscle Cells

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Abstract

While growth of blood vessels is important in hypertension, relatively little is known about the contribution of catecholamines. Using isolated rat aorta and cultured smooth muscle cells, we examined adrenergic stimulation of gene expression. Phenylephrine, a selective alpha$_1$ adrenergic receptor agonist, caused a rapid and transient increase in c-*fos* mRNA accumulation which was inhibited by prazosin, an alpha$_1$ receptor antagonist. Similarly, phenylephrine stimulated c-*jun* and c-*myc* mRNA accumulation. Chlorethylclonidine, a compound which irreversibly blocks alpha$_{1B}$ receptors, completely blocked the phenylephrine-induced increase in c-*fos* mRNA. RNase protection experiments demonstrated that rat aorta prominently expressed mRNA for alpha$_{1B}$ and alpha$_{1A/D}$ receptors. Phenylephrine-induced c-*fos* mRNA was partially inhibited by H-7, a protein kinase C inhibitor, and by nifedipine, a Ca^{2+} channel blocker; these two compounds together had additive effects. In situ hybridization showed that expression of c-*fos* mRNA induced by phenylephrine was localized to aorta's medial layer. These results suggest that alpha$_1$ receptor–induced increase in c-*fos* mRNA in aorta is mediated by a chlorethylclonidine-sensitive receptor subtype signaling via increasing intracellular Ca^{2+} concentrations and activating protein kinase C. (*J. Clin. Invest.* 1994. 94:210–218.) Key words: alpha$_1$ adrenergic receptors • c-*fos* • vascular smooth muscle • gene expression

Introduction

Catecholamines, such as epinephrine and norepinephrine, regulate blood pressure via rapid and reversible effects on contraction of blood vessels by activating alpha adrenergic receptors in vascular smooth muscle cells (VSMC)[1] (1). These effects are mediated mainly by alpha$_1$ adrenergic receptors (2). Two major subtypes of alpha$_1$ receptors (alpha$_{1A}$ and alpha$_{1B}$) have been identified in pharmacological studies (3–5). Among several criteria, alpha$_{1A}$ receptors are insensitive to the irreversible alpha adrenergic antagonist chlorethylclonidine, whereas alpha$_{1B}$ receptors are inactivated by chlorethylclonidine (2). The cDNA encoding the alpha$_{1B}$ adrenergic receptor from hamster DDT$_1$MF$_2$ cells has been cloned (6). An additional subtype, termed the alpha$_{1C}$ receptor, has been cloned from bovine brain; the pharmacological significance of this subtype is uncertain (7). Subsequently, two groups cloned an additional subtype of alpha$_1$ adrenergic receptors termed alpha$_{1A}$ (8) or alpha$_{1D}$ (9); the pharmacological properties of this receptor are similar but significantly different from those of the pharmacologically defined alpha$_{1A}$ receptor. Consequently, it appears that the pharmacologically described alpha$_{1B}$ receptor has been identified by molecular cloning, whereas the pharmacologically identified alpha$_{1A}$ receptor has not been isolated yet by molecular biological techniques.

In addition to regulation of blood vessel contraction, catecholamines also have long-term trophic effects on the growth of arterial smooth muscle cells. Epinephrine and norepinephrine stimulate the proliferation of rat thoracic and bovine aortic smooth muscle cells in culture (10, 11). Nakaki et al. (12) reported that norepinephrine stimulates DNA synthesis in quiescent cloned rat aortic smooth muscle cells. Prazosin, a selective alpha$_1$ adrenergic receptor antagonist, reduces intimal hyperplasia in the rabbit abdominal aorta (13). There is evidence suggesting that catecholamine receptors in vascular smooth muscle induce c-*fos* gene and stimulate cell growth (14). By injecting adrenergic agonists into rats, Majesky et al. (14) showed that alpha$_1$ adrenergic stimulation caused an increase in c-*fos* mRNA in rat aorta. c-*fos* is a protooncogene which likely plays an important role in cell growth; protooncogenes have been shown to be activated early during the development of smooth muscle cell hypertrophy (15).

The purpose of this study was to investigate the potential expression and role of alpha$_1$ adrenergic receptor subtypes in rat aorta and cultured rat aortic smooth muscle cells in inducing the expression of c-*fos* gene. It is not known which subtypes of alpha$_1$ adrenergic receptors are involved in the growth of smooth muscle cells. Using an in vitro preparation of rat aorta and cultured VSMC, we investigated and compared alpha$_1$ adrenergic induction of c-*fos* mRNA.

Methods

Materials. Agarose, trypsin-EDTA (×10), antibiotic antimycotic (×100) including 10,000 U penicillin, 10,000 μg streptomycin, and 25 μg amphotericin B/ml, 1:1 mixture of DME and Ham's F12, L-glutamine, and RNase T_2 were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum was from Hyclone Laboratories (Logan, UT). Guanidine thiocyanate, formamide, and restriction enzymes were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). Phenylephrine HCl, isoproterenol HCl, propranolol HCl, nifedipine, angiotensin II, H-7 (1-[5-isoquinolinylsulfonyl]-2-methylpiperazine), yeast total RNA (type XI), and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO). Chlorethylclonidine was

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1. *Abbreviations used in this paper:* CEC, chlorethylclonidine; VSMC, vascular smooth muscle cells.

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from Research Biochemicals Inc. (Natick, MA). RNA polymerases (T3, T7, and SP6), DNase I, and pGEM-3Z vector were from Promega Corp. (Madison, WI). pBluescript KS (+) vector was from Stratagene Inc. (La Jolla, CA). Collagenase (CLS IV) was from Worthington Biochemicals Corp. (Freehold, NJ). Elastase was from Elastin (Pacific, MO). Bisindolylmaleimide GF 109203X was from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Prazosin HCl was a gift from Pfizer (New York). Bovine serum albumin was from Calbiochem-Novabiochem Corp. (La Jolla, CA). v-*fos* genomic DNA (pfos-1), mouse c-*jun* cDNA (JAC.1), and human β-actin cDNA (HHCI89) were from American Type Culture Collection (Rockville, MD). v-*myc* genomic DNA was from Clontech Laboratories, Inc. (Palo Alto, CA). Random primer labeling system, deoxycytidine 5′-[α-^{32}P]triphosphate, cytidine 5′-α-[^{35}S]thiotriphosphate, and uridine 5′-[α-^{32}P]triphosphate were from Amersham Corp. (Arlington Heights, IL). OCT compound was from Miles Laboratories Inc. (Elkhart, IN). Other chemicals were from standard commercial sources.

Tissue preparation. Tissue preparation of rat aorta was performed as described by Hiremath et al. (16). Briefly, thoracic aortas from male Sprague-Dawley rats (200–250 g) were dissected out, and fat and connective tissues were removed. The vessels were placed in KRB at 37°C and were equilibrated for 60 min while bubbled with 95% O_2/5% CO_2. Then, vessels were incubated with various drugs as described.

Cell culture. VSMC (medial layer) and fibroblasts (adventitia layer) were prepared in culture by enzyme digestion of aorta from male Sprague-Dawley rats (200–250 g) by modification of the procedure of Smith and Brock (17). Cultured cells were maintained in a 1:1 mixture of DME and Ham's F12 (DME/F12), 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), 2 mM L-glutamine, and 15 mM Hepes. Cells were incubated at 37°C in humidified atmosphere of 95% air/5% CO_2. The medium was changed every 3 d, and cells were passaged by 0.05% trypsin/0.02% EDTA solution. Studies were conducted on VSMC (passages 4–12) and fibroblasts (passages 3–8) that had achieved confluence in 10% FCS/DME/F12. Then, the cells were incubated in a defined serum-free medium (DME/F12 plus insulin [5×10^{-7} M], transferrin [5 μg/ml], and ascorbic acid [0.2 mM]) for 2 d before addition of drugs as described.

RNA isolation and Northern blotting. Total RNA was extracted essentially as described by Chomczynski and Sacchi (18). Total RNA was fractionated by 1% agarose gel electrophoresis and transferred to a nylon filter by capillary action. ^{32}P-labeled DNA probes of v-*fos* (1.0-kb PstI fragment), c-*jun* (1.8-kb EcoRI-PstI fragment), v-*myc* (1.52-kb PstI fragment), and human β-actin (1.1-kb EcoRI fragment) were made with deoxycytidine 5′-[α-^{32}P]triphosphate by random primer labeling according to the manufacturer's instructions. After baking, the filter was hybridized in 50% formamide, 5 × SSPE buffer, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA at 42°C for 16–20 h. The filter was washed twice with 2 × SSC, 0.1% SDS at 60°C (c-*fos*, c-*jun*, and c-*myc*) or at 65°C (β-actin) for 15 min and with 0.1 × SSC, 0.1% SDS at 60°C (c-*fos*, c-*jun*, and c-*myc*) or at 65°C (β-actin) for 15–30 min and was exposed to Kodak XAR-5 film at −70°C with intensifying screen. The autoradiograms were scanned using a densitometer.

RNase protection assay. Plasmid containing 188-bp fragment of rat alpha$_{1A/D}$ receptor cDNA (1833/2021) was produced by cutting ApaI sites of alpha$_{1A/D}$ receptor cDNA and subcloning this fragment into HindIII/ApaI sites of pBluescript KS (+) vector. Plasmid containing 306-bp fragment of rat alpha$_{1B}$ receptor cDNA (910/1216) was produced by cutting PstI/BamHI sites of rat alpha$_{1B}$ receptor cDNA and plasmid DNA and subcloning this fragment into PstI/BamHI sites of pGEM-3Z vector. A 165-bp fragment of rat β-actin cDNA (695/860) was produced by PCR with oligos AAAGCTTGAGGGAAATCGTGCGTGACAT and GGAATTCAGGAAGGAAGGCTGGAAGAGA (sequences underlined are linkers for providing sites for EcoRI and HindIII) as 5′- and 3′-primers according to a published rat β-actin cDNA sequence (19). The appropriately sized PCR product was subcloned into EcoRI and HindIII sites of pBluescript KS (+) vector and used as an internal control.

Total RNAs were prepared as described above, and a modified RNase protection assay was performed according to recently reported methods (20, 21). Plasmids with alpha$_{1A/D}$ and alpha$_{1B}$ receptor probe sequences obtained as described above were linearized with HindIII or EcoRI; the plasmid containing the β-actin probe sequence was linearized with EcoRI. Radiolabeled RNA probes were produced by incubating the plasmid that contained cDNA fragments from the rat alpha$_{1A/D}$ or alpha$_{1B}$ receptor cDNAs with T7/SP6 RNA polymerases and from the β-actin cDNA with T3 RNA polymerase in the presence of uridine 5′-[α-^{32}P]triphosphate (100 μCi) at 37°C for 1 h. The alpha$_{1A/D}$ or alpha$_{1B}$ receptor RNA probe (5×10^5 cpm) with the β-actin RNA probe (0.5–1×10^5 cpm) as an internal control and 50 μg of total RNA were mixed and hybridized at 55°C for 30 h in hybridization buffer (80% formamide, 0.4 M NaCl, 50 mM Pipes, and 1 mM EDTA). RNase buffer containing 30 U/ml RNase T_2 was added to each assay tube and then incubated for 60 min at 30°C. RNase-resistant hybrids were precipitated, run on a 6% polyacrylamide/8 M urea gel, and exposed to Kodak XAR-5 film at −70°C with intensifying screen. The size of fragments protected was estimated from positions of DNA molecular marker which was 5′-end labeled with [γ-^{32}P]ATP and run in the same gel.

In situ hybridization. In situ hybridization was performed using a ^{35}S-labeled RNA probe specific for rat c-*fos* according to the method described by Bradley et al. (22) and Makeover et al. (23) with a few modifications. The aortas were fixed with chilled 4% paraformaldehyde in PBS for 4 h, incubated with 30% sucrose in PBS for 2 h, and embedded in OCT compound. Frozen sections (10 μm) were acetylated with acetic anhydride and dehydrated in serial alcohol (22). Both sense and antisense single-stranded radiolabeled RNA probes were produced by incubating cytidine 5′-α-[^{35}S]thiotriphosphate (50 μCi) and the linearized plasmid containing a 232-bp fragment (356/588) of rat c-*fos* cDNA using either T3 or T7 RNA polymerase at 37°C for 1 h. The sections were hybridized in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl, 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 100 mM DTT, 0.1% SDS, 0.1 mg/ml salmon sperm DNA, 0.25 mg/ml yeast total RNA, 0.25 mg/ml yeast transfer RNA, and 2×10^7 cpm/ml of the ^{35}S-labeled probe for 20–24 h at 50°C. The sections were washed twice in 2× SSC/50% formamide containing 0.1% β-mercaptoethanol at 50°C for 15 min, digested with 20 μg/ml RNase A at 37°C for 30 min, and washed three times in 2× SSC/50% formamide containing 0.1% β-mercaptoethanol at 50°C for 15 min. The sections were dipped in Kodak NTB-2 emulsion and exposed for 3 wk. After development, the sections were counterstained with hematoxylin and eosin and visualized with dark-field or bright-field microscopy.

Results

The time course of effects of the selective alpha$_1$ agonist phenylephrine on c-*fos* mRNA values in rat aorta in vitro is shown in Fig. 1 *A*. In the absence of phenylephrine, c-*fos* mRNA values remained constant for the duration of the experiment. In contrast, continuous incubations of aorta with phenylephrine (10^{-5} M) resulted in an early and transient increase in c-*fos* mRNA. c-*fos* mRNA increased from 30 min and reached a maximal value at 60 min after the addition of phenylephrine. After 240 min, the accumulation of c-*fos* mRNA decreased to values close to basal. The dose-dependent effect of phenylephrine on c-*fos* mRNA values after a 1-h activation of aortas is shown in Fig. 1 *B*. Phenylephrine enhanced accumulation of c-*fos* mRNA in a dose-dependent fashion. Phenylephrine's 50% effective concentration was $\sim 3 \times 10^{-7}$ M. Both β-actin mRNA (2.2 kb) and α-actin mRNA (1.7 kb) were detected with the β-

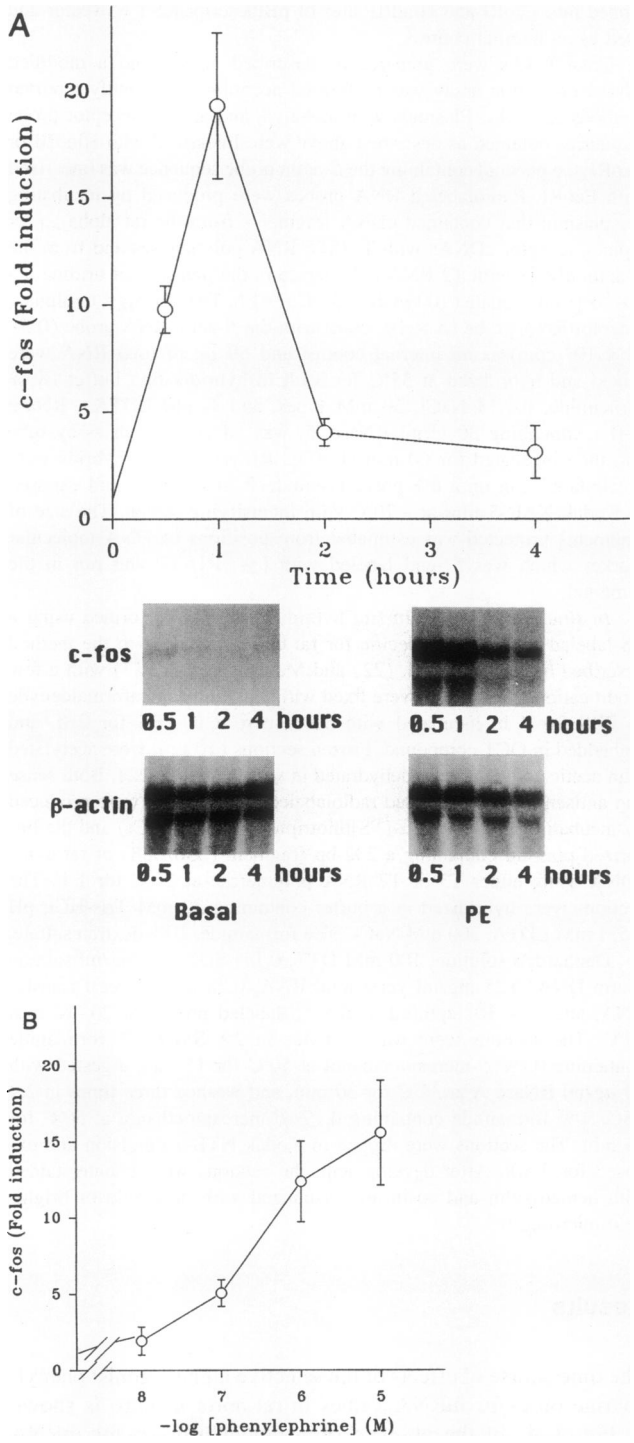

Figure 1. Phenylephrine-induced c-*fos* mRNA in rat aorta. (*A*) Time course. Rat aortas were incubated at 37°C for the times indicated in the presence of phenylephrine (10^{-5} M). (*B*) Concentration–response curve. Aortas were stimulated for 1 h at 37°C with various concentrations of phenylephrine. The c-*fos* mRNA values were analyzed by Northern blotting. Using β-actin cDNA, β-actin mRNA (2.2 kb), and α-actin mRNA (1.7 kb) were detected. β-Actin mRNA was used as an internal control. Data, expressed as fold increase over the control at each time point, are the means±SEM of three separate experiments. Panels in *A* are representative Northern blots of c-*fos* mRNAs and β-actin mRNAs. Rat aortas were incubated at 37°C for the times indicated in the absence (*Basal*) or the presence of phenylephrine (*PE*) (10^{-5} M).

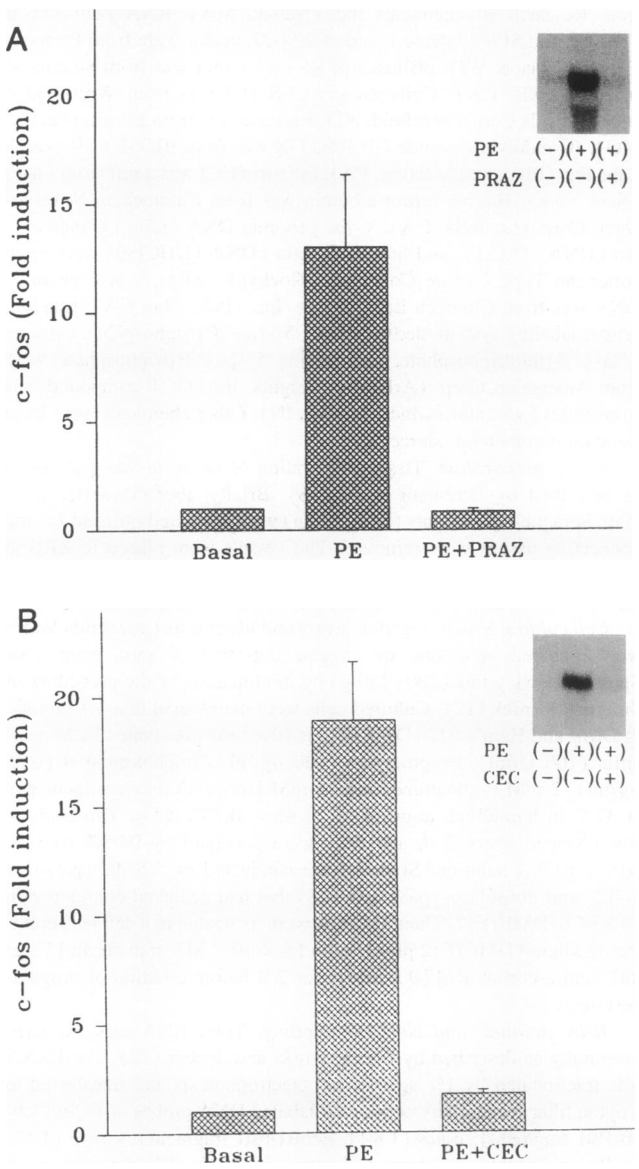

Figure 2. Effects of alpha$_1$ antagonists on phenylephrine-induced c-*fos* mRNA in rat aorta. (*A*) Rat aortas were stimulated for 1 h at 37°C with phenylephrine (*PE*) (10^{-5} M) in the absence or the presence of prazosin (*PRAZ*) (10^{-6} M). Prazosin was added 15 min before phenylephrine administration. (*B*) Rat aortas were incubated with CEC (10^{-4} M) for 30 min at 37°C. After washing four times with KRB, the vessels were equilibrated for 30 min and were stimulated with phenylephrine (*PE*) (10^{-5} M) for 1 h. The c-*fos* mRNA values were analyzed by Northern blotting. β-Actin mRNA was used as an internal control. Data, expressed as fold increase over the control, are the means±SEM of three separate experiments. Insets are representative Northern blots of c-*fos* mRNAs.

actin cDNA used as a hybridization probe (14). β-Actin mRNAs were unchanged by phenylephrine.

Effects of adrenergic receptor antagonists on phenylephrine-induced c-*fos* mRNA in rat aorta are shown in Fig. 2, *A* and *B*. In aorta, induction of c-*fos* mRNA by phenylephrine (10^{-5} M) was abolished by the alpha$_1$ adrenergic receptor antagonist prazosin (10^{-6} M). The beta receptor antagonist propranolol (10^{-5} M) did not inhibit the induction by phenylephrine (data

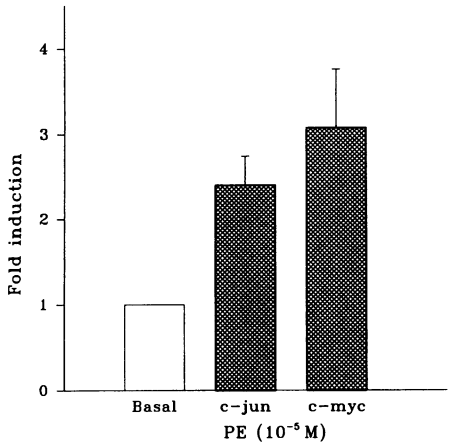

Figure 3. Effect of phenylephrine on c-*jun* and c-*myc* mRNA in rat aorta. Rat aortas were stimulated for 1 h (c-*jun*) and for 4 h (c-*myc*) at 37°C with phenylephrine (*PE*) (10^{-5} M). The c-*jun* and c-*myc* mRNA values were analyzed by Northern blotting. Data, expressed as fold increase over the basal, are the means±SEM (*bar*) of three separate experiments. Induction of c-*jun* mRNA by phenylephrine was inhibited by prazosin in each of three experiments (data not shown). Induction of c-*myc* mRNA by phenylephrine was inhibited by prazosin which was tested in one experiment (data not shown).

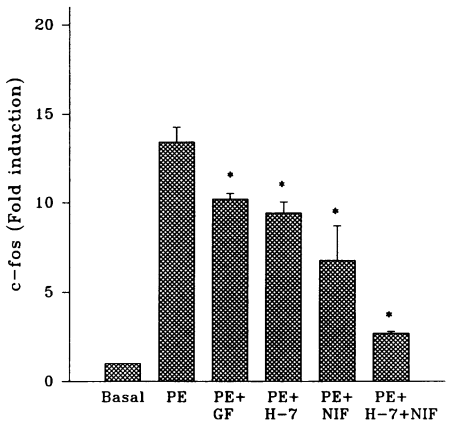

Figure 4. Effects of H-7, bisindolylmaleimide GF 109203X, and nifedipine on phenylephrine-induced c-*fos* mRNA in rat aorta. Rat aortas were stimulated for 1 h at 37°C with phenylephrine (*PE*) (10^{-5} M) in the absence or the presence of H-7 (10^{-5} M), bisindolylmaleimide GF 109203X (*GF*) (10^{-6} M), or nifedipine (*NIF*) (10^{-6} M). These compounds were added 1 h before phenylephrine administration. The c-*fos* mRNA values were analyzed by Northern blotting. β-Actin mRNA was used as an internal control. Data, expressed as fold increase over the control, are the means±SEM of three separate experiments. $*P < 0.05$ vs PE. Statistical analysis was performed using a Student's *t* test.

not shown). These results suggest that in aorta alpha$_1$ adrenergic receptors mediate phenylephrine-induced c-*fos* mRNA. To examine which subtype(s) of alpha$_1$ adrenergic receptors mediates phenylephrine-induced c-*fos* mRNA, we investigated the effects of chlorethylclonidine (CEC), a drug which has been used as an irreversible alpha$_{1B}$ adrenergic receptor antagonist. Rat aortas were incubated with CEC (10^{-4} M) for 30 min, were washed with KRB four times, and then were equilibrated for 30 min in buffer before stimulation with phenylephrine. As shown in Fig. 2 *B*, CEC completely blocked phenylephrine-induced c-*fos* mRNA. This result indicates that phenylephrine-induced c-*fos* mRNA was mediated by a CEC-sensitive alpha$_1$ adrenergic receptor subtype in rat aorta.

We investigated the effect of phenylephrine on c-*jun* and c-*myc* mRNA values in rat aorta (Fig. 3). Phenylephrine caused increases in c-*jun* and c-*myc* mRNA which were both inhibited by prazosin (data not shown). Fold inductions of c-*jun* and c-*myc* mRNA from basal values were less than those which occurred for c-*fos* mRNA.

To examine the second messenger systems involved in these effects of alpha$_1$ receptors on expression of c-*fos* mRNA, we investigated the actions of H-7 and bisindolylmaleimide GF 109203X, protein kinase C inhibitors, and nifedipine, an L-type Ca^{2+} channel antagonist, on phenylephrine-induced c-*fos* mRNA in rat aorta. The induction of c-*fos* mRNA by phenylephrine was partially inhibited by either H-7 (10^{-5} M), bisindolylmaleimide GF 109203X (10^{-6} M), or nifedipine (10^{-6} M) (Fig. 4). H-7 and nifedipine together had an additive effect on suppression of phenylephrine-induced c-*fos* mRNA accumulation. Neither H-7, bisindolylmaleimide GF 109203X, nor nifedipine alone had any effect on basal c-*fos* mRNA values (data not shown).

The time course of effects of phenylephrine on c-*fos* mRNA values in cultured rat aortic smooth muscle cells is shown in Fig. 5 *A*. In the absence of phenylephrine, c-*fos* mRNA values remained constant for the duration of the experiment (data not shown). In contrast, continuous incubations of VSMC with phenylephrine (10^{-5} M) resulted in an early and transient increase in c-*fos* mRNA. c-*fos* mRNA increased from 15 min and achieved a maximal value at 30 min after the addition of phenylephrine. After 120 min, the accumulation of c-*fos* mRNA value decreased to close to basal values. Compared with rat aorta, in VSMC c-*fos* mRNA values reached a maximal value more quickly. The dose-dependent effect of phenylephrine on c-*fos* mRNA value after 30 min of treatment in VSMC is shown in Fig. 5 *B*. Phenylephrine's 50% effective concentration was $\sim 6 \times 10^{-7}$ M.

Effects of adrenergic receptor antagonists on phenylephrine-induced c-*fos* mRNA in rat VSMC are shown in Fig. 6, *A* and *B*. In VSMC, induction of c-*fos* mRNA by phenylephrine (10^{-5} M) was abolished by prazosin (10^{-6} M), while propranolol (10^{-5} M) did not inhibit the induction by phenylephrine (data not shown), similar to what was seen previously in rat aorta. These results suggest that induction of c-*fos* mRNA by phenylephrine is mediated through alpha$_1$ adrenergic receptors. Cultured cells were incubated with CEC for 30 min, were washed with DME/F12 medium four times, and were equilibrated for 30 min in medium before the stimulation of phenylephrine. CEC blocked phenylephrine-induced c-*fos* mRNA completely. This result indicates that phenylephrine-induced c-*fos* mRNA is mediated by CEC-sensitive alpha$_1$ adrenergic receptors in VSMC as well as in rat aorta.

We compared increases in c-*fos* mRNA values induced by phenylephrine and angiotensin II in Fig. 7, *A* and *B*. In aorta, induction of c-*fos* mRNA by phenylephrine was much greater than that mediated by angiotensin II. On the other hand, in VSMC the effect of angiotensin II (10^{-6} M) on expression of c-*fos* mRNA was much greater than that of phenylephrine (10^{-5} M), the opposite of the result found in intact rat aorta incubated in vitro with these agonists.

We prepared adventitia fibroblasts in culture from the outer layer of aorta and investigated effects of adrenergic agents on

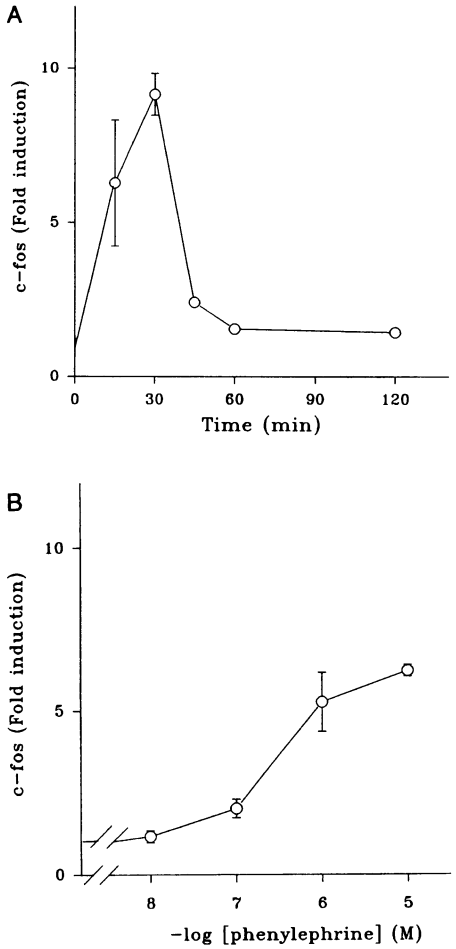

Figure 5. Phenylephrine-induced c-*fos* mRNA in cultured rat VSMC. (*A*) Time course. Smooth muscle cells were incubated at 37°C for the times indicated in the presence of phenylephrine (10^{-5} M). (*B*) Concentration–response curve. Cells were stimulated for 30 min at 37°C with various concentrations of phenylephrine. The c-*fos* mRNA values were analyzed by Northern blotting. β-Actin mRNA was used as an internal control. Data, expressed as fold increase over the control, are the means±SEM of three separate experiments.

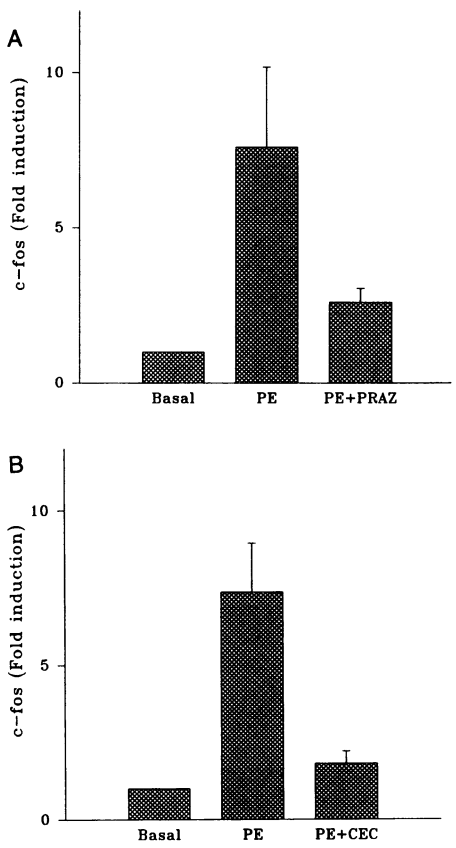

Figure 6. Effects of alpha$_1$ antagonists on phenylephrine-induced c-*fos* mRNA in cultured rat VSMC. (*A*) Cells were stimulated for 30 min at 37°C with phenylephrine (*PE*) (10^{-5} M) in the absence or the presence of prazosin (*PRAZ*) (10^{-6} M). Prazosin was added 15 min before phenylephrine administration. (*B*) Cells were incubated with CEC (10^{-4} M) for 30 min at 37°C. After washing four times with DME-F12 medium, cells were equilibrated for 30 min and were stimulated with phenylephrine (10^{-5} M) for 30 min. The c-*fos* mRNA values were analyzed by Northern blotting. β-Actin mRNA was used as an internal control. Data, expressed as fold increase over the control, are the means±SEM of three separate experiments.

c-*fos* mRNA using these cells. In adventitia fibroblasts, beta adrenergic stimulation induced c-*fos* mRNA, whereas alpha$_1$ adrenergic stimulation had little effect (data not shown).

Because aortic tissue represents a heterogeneous cell population, in situ hybridization analysis was performed to directly demonstrate localization of c-*fos* mRNA (Fig. 8). Emulsion autoradiography sections demonstrated that basal expression of c-*fos* mRNA was low and that increased expression after activation with phenylephrine was seen primarily in the medial smooth muscle layer rather than in the adventitial fibroblast layer. Only a few grains were visible over sections of aorta which were hybridized to the sense probe, suggesting low nonspecific binding.

Using an RNase protection assay, we detected alpha$_{1A/D}$ adrenergic receptor mRNA as Lomasney et al. (8) did previously using Northern blotting (Fig. 9). In addition, we detected alpha$_{1B}$ adrenergic receptor mRNA as well which had not been detected previously in Northern blotting experiments. These RNase protection experiments were not designed to compare the relative expression of these subtypes in rat aorta.

Discussion

This study demonstrates that in intact rat aorta incubated in vitro and in cultured rat VSMC, phenylephrine induces a rapid and transient increase in c-*fos* mRNA which is blocked by prazosin. The increase in c-*fos* mRNA induced by phenylephrine was also blocked by chlorethylclonidine. The protein kinase C inhibitor H-7 and L-type Ca^{2+} channel antagonist nifedipine each partially inhibited phenylephrine-induced c-*fos* mRNA expression. The relative response to activation of alpha$_1$ adrenergic receptors versus angiotensin II receptors was much greater in aorta compared with cultured VSMC.

While it is well known that the sympathetic nervous system has marked effects on blood vessel contraction, autonomic nerves also have trophic effects on arterial smooth muscle mass (1, 24–26). Catecholamines stimulate the growth of arterial smooth muscle cells in vitro (27). Majesky et al. (14) reported

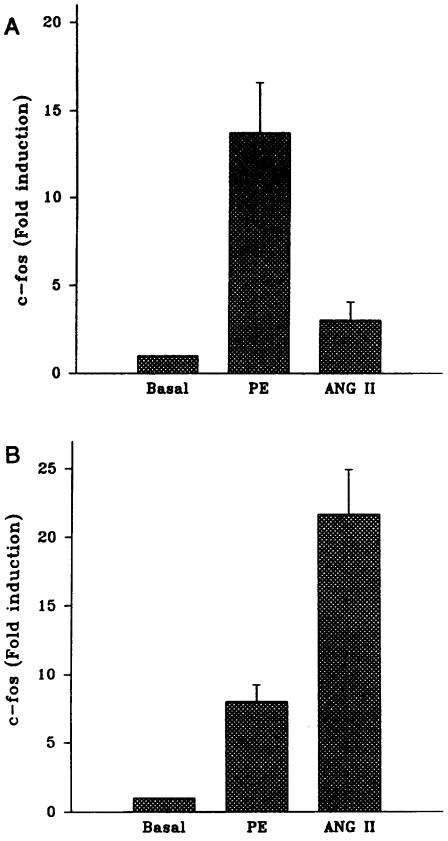

Figure 7. Comparison of effect of angiotensin II on c-*fos* mRNA with that of phenylephrine in rat aorta and cultured rat VSMC. (*A*) Rat aortas were stimulated for 1 h at 37°C with or without phenylephrine (*PE*) (10^{-5} M) or angiotensin II (*ANG II*) (10^{-6} M). (*B*) Cultured cells were stimulated for 30 min at 37°C with or without phenylephrine (10^{-5} M) or angiotensin II (10^{-6} M). The c-*fos* mRNA values were analyzed by Northern blotting. β-Actin mRNA was used as an internal control. Data, expressed as fold increase over the control, are the means±SEM of three separate experiments.

that alpha$_1$ adrenergic stimulation causes induction of growth-related genes including platelet-derived growth factor A-chain and c-*fos* in aorta of intact rats. Furthermore, in isolated heart and cultured cardiomyocytes, alpha$_1$ adrenergic stimulation induces c-*fos* mRNA (28, 29). It has been suggested that the c-*fos* gene, which is induced by platelet-derived growth factor, angiotensin II, and endothelin, has an important influence on the growth of vascular smooth muscle (30–33).

Studies of alpha$_1$ adrenergic receptor–mediated contraction of various smooth muscles reveal evidence for subtypes of alpha$_1$ adrenergic receptors. Minneman et al. (4) showed, in studies examining several rat tissues, the proportion of alpha$_1$ adrenergic receptor–binding sites inactivated by chlorethylclonidine correlated with the sites having a low affinity for WB4101. They proposed the existence of two alpha$_1$ adrenergic receptor subtypes, which were termed alpha$_{1A}$ (chlorethylclonidine-insensitive) and alpha$_{1B}$ (chlorethylclonidine-sensitive) (4). The CEC-sensitive and -insensitive alpha$_1$ adrenergic receptors are equivalent to those with a low and high affinity for WB4101, respectively (4). The pharmacological characteristics of the alpha$_{1A}$ and alpha$_{1B}$ adrenergic receptor subtypes suggest that several tissues or cell lines predominantly express one of the subtypes. Rat liver, rat spleen, DDT_1MF_2 cells (a hamster smooth muscle cell line), and FRTL cells (a rat thyroid cell line) appear to contain almost exclusively the alpha$_{1B}$ receptor subtype (34). In contrast, rat renal artery contains primarily alpha$_{1A}$ adrenergic receptors, while any tissues have significant numbers of each of these receptor subtypes (34).

The molecular cloning of several alpha$_1$ adrenergic receptor subtypes has been reported (6–9). The alpha$_1$ adrenergic receptor expressed in DDT_1MF_2 cells was the first subtype cloned and has the expected properties of an alpha$_{1B}$ adrenergic receptor described pharmacologically (6). An alpha$_1$ adrenergic receptor cDNA isolated from a bovine brain which does not have the characteristics of an alpha$_{1A}$ or alpha$_{1B}$ receptor subtype has been termed the alpha$_{1C}$ subtype (7). Subsequently, two groups cloned additional alpha$_1$ adrenergic cDNAs from brain which have similar properties to that of the pharmacologically described alpha$_{1A}$ subtype, but not exactly (8, 9). These cDNAs are identical except for two codons and are likely the same receptor subtype which has been recently termed the alpha$_{1A/D}$ receptor (35) although no consistent terminology has been settled.

To investigate which subtypes of alpha$_1$ adrenergic receptors mediate induction of c-*fos* mRNA, we used CEC, which selectively and irreversibly inactivates alpha$_{1B}$ receptors in pharmacological experiments. In addition, CEC may at least partially block cloned and expressed alpha$_{1A/D}$ receptors (8, 9). Alpha$_{1C}$ receptors may be partially resistant to CEC (7). In other words, CEC fully inactivates alpha$_{1B}$ receptors and partially blocks the cloned alpha$_{1A/D}$ and alpha$_{1C}$ receptors; on the other hand, CEC spares the pharmacologically defined alpha$_{1A}$ receptor which may not have yet been cloned. We found that CEC completely blocked phenylephrine-induced c-*fos* mRNA accumulation. This result suggests that in rat aorta induction of c-*fos* mRNA was mediated by alpha$_{1B}$ adrenergic receptors or possibly also alpha$_{1A/D}$ receptors. These results also suggest that the pharmacologically defined CEC-insensitive alpha$_{1A}$ receptor does not regulate expression of c-*fos* mRNA in rat aorta. In rat tissues, the major alpha$_1$ subtypes are alpha$_{1A}$ and alpha$_{1B}$ defined pharmacologically (34). Experiments measuring catecholamine-induced contraction of rat aorta based on sensitivity to CEC suggest that alpha$_{1B}$ adrenergic receptors are the subtype predominantly mediating contraction in this vessel (36). However, norepinephrine-induced contraction of rat aorta is also inhibited by WB4101, a putative selective alpha$_{1A}$ adrenergic receptor antagonist (36). Thus, subclassification of alpha$_{1A}$ and alpha$_{1B}$ adrenergic receptors in rat aorta in terms of contractile responses by potentially selective compounds is uncertain.

Lomasney et al. (8) used both alpha$_{1B}$ and alpha$_{1A/D}$ adrenergic receptor cDNA probes to investigate expression of these subtypes in rat aorta using Northern blot analysis and detected only alpha$_{1A/D}$ adrenergic receptor mRNA. However, as indicated above, functional studies in rat aorta have demonstrated that the selective irreversible alpha$_{1B}$ adrenergic receptor antagonist CEC markedly inhibits the contraction induced by the stimulation of alpha$_1$ adrenergic receptors in rat aorta (36, 37), suggesting that alpha$_{1B}$ adrenergic receptors are expressed in rat aorta. Indeed, we have found in the current study using an RNase protection assay that both alpha$_{1B}$ and alpha$_{1A/D}$ mRNAs are expressed in rat aorta. As RNase protection assays are generally more sensitive and specific than Northern blot analysis, those data provide strong evidence for expression of both these receptor subtypes in rat aorta. We also found faint alpha$_{1C}$ recep-

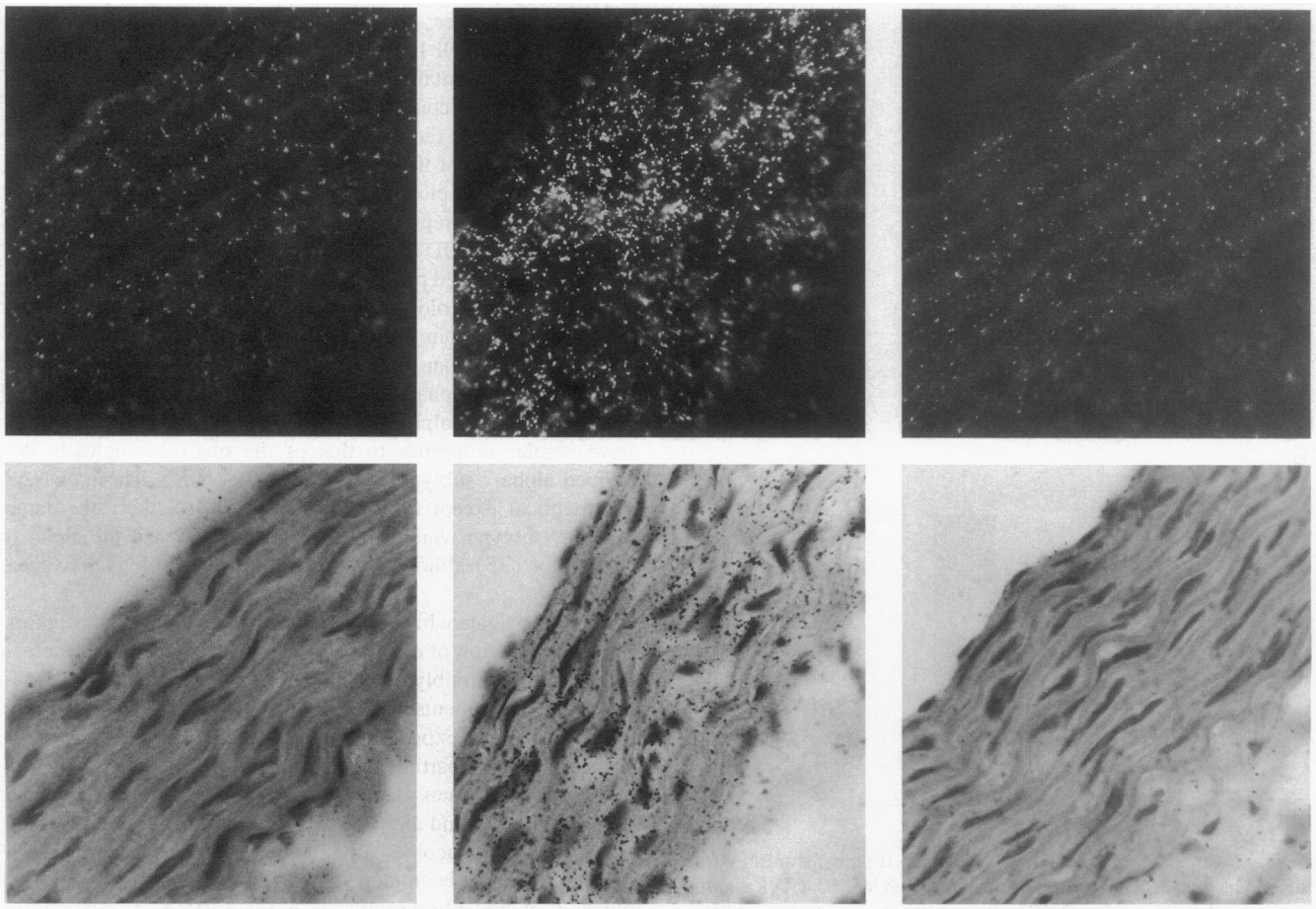

Figure 8. Photographs showing representative in situ hybridization analysis of phenylephrine-induced c-*fos* mRNA in aorta. Rat aortas were incubated for 1 h at 37°C in the absence or the presence of phenylephrine (10^{-5} M). Nonstimulated aorta (*left panels*) and stimulated aorta with phenylephrine (*middle panels*) were fixed and OCT-embedded, and sections were hybridized using a ^{35}S-labeled antisense RNA probe specific for rat c-*fos* as described in Methods. To evaluate nonspecific background, aorta stimulated with phenylephrine was hybridized using a ^{35}S-labeled sense RNA probe specific for rat c-*fos* (*right panels*). After RNase digestion and washing, sections underwent emulsion autoradiography, were counterstained with hematoxylin and eosin, and visualized with dark-field microscopy (*upper panels*) or with bright-field microscopy (*lower panels*). In all photographs, endothelial layers are to the left-hand side. Data are representative of three separate experiments. ×400.

tor mRNA signals in aorta, suggesting the possibility of low levels of expression of that subtype in aorta as well (data not shown).

Efforts have been extended to link the various alpha$_1$ receptor subtypes to specific signal transduction mechanisms. Activation of alpha$_{1A}$ adrenergic receptors have been found to promote the influx of extracellular Ca^{2+} via L-type Ca^{2+} channels (38). In contrast, alpha$_{1B}$ and alpha$_{1C}$ adrenergic receptors are linked to stimulation of phospholipid hydrolysis which produces two second messengers, diacylglycerol, which activates protein kinase C, and inositol 1,4,5-triphosphate, which acts on a specific intracellular receptor to release sequestered Ca^{2+} (35, 38). Recently, it has been shown that cloned and expressed alpha$_{1A/D}$ adrenergic receptors activate influx of extracellular Ca^{2+} through L-type Ca^{2+} channels (35). However, it is difficult and in any case premature to classify the alpha adrenergic receptor subtypes on the basis of their potential signaling mechanisms. Inhibition of phenylephrine-induced c-*fos* accumulation by the two protein kinase C inhibitors H-7 and bisindolylmaleimide GF 109203X (39) in rat aorta suggests that CEC-sensitive alpha$_1$ adrenergic receptors are coupled to stimulation of phospholipid hydrolysis, and activation of protein kinase C plays an important role in induction of c-*fos* mRNA. Furthermore, we have found that nifedipine also inhibits phenylephrine-induced c-*fos* mRNA accumulation. This result suggest that c-*fos* mRNA induced by phenylephrine is also dependent on the influx of extracellular Ca^{2+}. The potential mechanisms by which alpha$_1$ receptors activate c-*fos* gene expression have considerable similarities with those for activation of contraction by these receptors. In rat aorta, mechanisms for activation of contraction by alpha$_1$ adrenergic receptors are also complex, involving several components having different time courses of action (40–42). Contraction occurs because of both Ca^{2+} influx and release of intracellular Ca^{2+} and activation of protein kinase C as a consequence of activation of phosphoinositide metabolism. For example, Nishimura et al. (42) found that nifedipine and H-7 independently partially inhibited phenylephrine-induced contraction in rat aorta, whereas both compounds together almost completely inhibited contraction. Interestingly, induction of c-*fos* mRNA by angiotensin II in cultured rat aortic smooth muscle cells is dependent on both a rise in intracellular Ca^{2+} concentration and activation of protein kinase C (32).

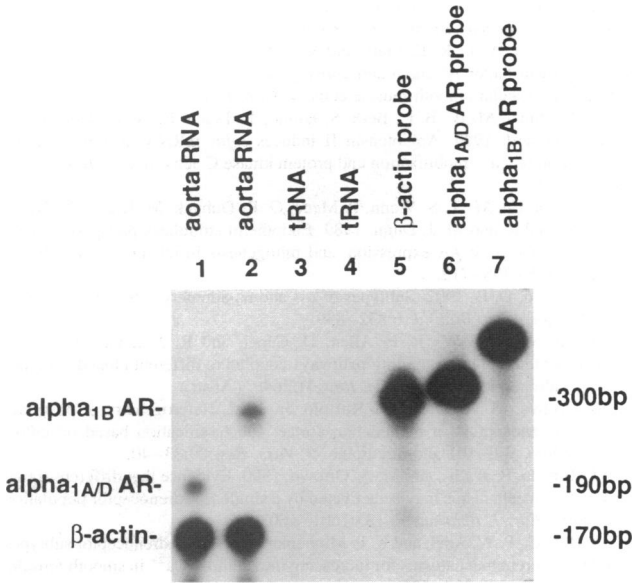

Figure 9. RNase protection assays of rat $alpha_{1A/D}$ and $alpha_{1B}$ adrenergic receptor mRNA in rat aorta. 50 μg of total RNA isolated from rat thoracic aorta (*aorta RNA*) or 10 μg of control transfer RNA (*tRNA*) was hybridized with RNA probes of $alpha_{1A/D}$ adrenergic receptor (*AR*) and β-actin (lanes *1* and *3*) or probes for $alpha_{1B}$ adrenergic receptor and β-actin (lanes *2* and *4*). After RNase digestion, RNase-resistant hybrids were run on a 6% polyacrylamide/8 M urea gel. Probes in the presence of tRNA were digested completely by RNase (tRNA lanes). Lanes *5–7* show undigested probes. Data are representative of three separate experiments.

In cultured smooth muscle cells, angiotensin II is known to be a strong inducer on c-*fos* mRNA accumulation and to be involved in vascular hypertrophy (15, 33). We compared the effect of phenylephrine on c-*fos* mRNA with that of angiotensin II both in rat isolated aorta and in cultured smooth muscle cells. In cultured VSMC, angiotensin II had a very large effect on induction of c-*fos* mRNA, much greater than seen with phenylephrine. In contrast, in rat aorta freshly isolated in vitro, the effect of phenylephrine was much greater than that of angiotensin II. The hypertrophic effect of angiotensin II in vitro is associated with the activation of protooncogenes such as c-*fos,* c-*jun,* and c-*myc* (43). We have shown that phenylephrine induces c-*jun* mRNA and c-*myc* mRNA as well as c-*fos* mRNA in rat aorta. This result suggests the possibility that adrenergic stimulation plays a very important role in vascular hypertrophy in intact aorta. Clearly, culturing of smooth muscle cells leads to changes in the responses to these agonists, which could be misleading if simply extrapolated back to the expected responses in intact vessels. The explanation for this change in culture is uncertain, and whether it relates to change in expression of $alpha_1$ adrenergic receptor subtypes or some other mechanism requires further investigation.

Rat aorta contains adventitia fibroblasts as well as smooth muscle. Results of the in situ hybridization experiments suggest that in rat aorta induction of c-*fos* mRNA via $alpha_1$ adrenergic receptors occurred primarily in the smooth muscle layer, which is consistent with the results in cultured VSMC and adventitia fibroblasts. These results suggest that in the intact rat aorta $alpha_1$ adrenergic stimulation of c-*fos* mRNA is due to response in medial smooth muscle and support the use of isolated rings of aorta as a useful model system to investigate growth-promoting effects of catecholamines.

Our results suggest that rat aorta expresses at least two $alpha_1$ adrenergic receptor subtypes ($alpha_{1A/D}$ and $alpha_{1B}$) and that these are potentially involved in inducing expression of c-*fos* gene in vascular smooth muscle. The signaling pathways involved in mediating these responses are complex, involving both Ca^{2+} influx and activation of protein kinase C. Isolated rings of aorta provide a useful model in the study of growth-promoting effects induced by catecholamines and may be particularly useful identifying novel pharmacological antagonists of these receptors.

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References

1. Folkow, B. 1982. Physiological aspects of primary hypertension. *Physiol. Rev.* 62:347–504.

2. Minneman, K. P. 1988. α_1-Adrenergic receptor subtypes, inositol phosphates and sources of cell Ca^{2+}. *Pharmacol. Rev.* 40:87–119.

3. Morrow, A. L., and I. Creese. 1986. Characterization of α_1-adrenergic receptor subtypes in rat brain: a reevaluation of [^{3}H]WB4101 and [^{3}H]prazosin binding. *Mol. Pharmacol.* 29:321–330.

4. Minneman, K. P., C. Han, and P. W. Abel. 1988. Comparison of α_1-adrenergic receptor subtypes distinguished by chlorethylclonidine and WB 4101. *Mol. Pharmacol.* 33:509–514.

5. Ruffolo, R. R., Jr., A. J. Nichols, J. M. Stadel, and J. P. Hieble. 1991. Structure and function of α-adrenoceptors. *Pharmacol. Rev.* 43:475–505.

6. Cotecchia, S., D. A. Schwinn, R. R. Randall, R. J. Lefkowitz, M. G. Caron, and B. K. Kobilka. 1988. Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA.* 85:7159–7163.

7. Schwinn, D. A., J. W. Lomasney, W. Lorenz, P. J. Szklut, R. T. Fremeau, Jr., T. L. Yang-Feng, M. G. Caron, R. J. Lefkowitz, and S. Cotecchia. 1990. Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype. *J. Biol. Chem.* 265:8183–8189.

8. Lomasney, J. W., S. Cotecchia, W. Lorenz, W.-Y. Leung, D. A. Schwinn, T. L. Yang-Feng, M. Brownstein, R. J. Lefkowitz, and M. G. Caron. 1991. Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor: the gene for which is located on human chromosome 5. *J. Biol. Chem.* 266:6365–6369.

9. Perez, D. M., M. T. Piascik, and R. M. Graham. 1991. Solution-phase library screening for the identification of rare clones: isolation of an α_{1D}-adrenergic receptor cDNA. *Mol. Pharmacol.* 40:876–883.

10. Blaes, N., and J.-P. Boissel. 1983. Growth-stimulating effect of catecholamines on rat aortic smooth muscle cells in culture. *J. Cell. Physiol.* 116:167–172.

11. Bell, L., and J. A. Madri. 1989. Effect of platelet factors on migration of cultured bovine aortic endothelial and smooth muscle cells. *Circ. Res.* 65:1057–1065.

12. Nakaki, T., M. Nakayama, S. Yamamoto, and R. Kato. 1990. α_1-Adrenergic stimulation and β_2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol. Pharmacol.* 37:30–36.

13. O'Malley, M. K., E. W. M. McDermott, D. Mehigan, and N. J. O'Higgins. 1989. Role for prazosin in reducing the development of rabbit intimal hyperplasia after endothelial denudation. *Br. J. Surg.* 76:936–938.

14. Majesky, M. W., M. J. A. P. Daemen, and S. M. Schwartz. 1990. α_1-Adrenergic stimulation of platelet-derived growth factor A-chain gene expression in rat aorta. *J. Biol. Chem.* 265:1082–1088.

15. Neyses, L., and H. Vetter. 1992. Molecular biology of oncogenes and cardiovascular hypertrophy. *J. Hypertens.* 10:1447–1452.

16. Hiremath, A. N., Z. W. Hu, and B. B. Hoffman. 1991. Desensitization of alpha-adrenergic receptor-mediated smooth muscle contraction: role of the endothelium. *J. Cardiovasc. Pharmacol.* 18:151–157.

17. Smith, J. B., and T. A. Brock. 1983. Analysis of angiotensin-stimulated sodium transport in cultured smooth muscle cells from rat aorta. *J. Cell. Physiol.* 114:284–290.

18. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

19. Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol. Cell. Biol.* 3:787–795.

20. Saccomanno, C. F., M. Bordonaro, J. S. Chen, and J. L. Nordstrom. 1992. A faster ribonuclease protection assay. *Biotechniques.* 13:846–850.

21. Hod, Y. 1992. A simplified ribonuclease protection assay. *Biotechniques.* 13:852–854.

22. Bradley, D. J., H. C. Towle, and W. S. Young III. 1992. Spatial and temporal expression of α- and β- thyroid hormone receptor mRNAs, including the β_2-subtype, in the developing mammalian nervous system. *J. Neurosci.* 12:2288–2302.

23. Makeover, A., D. R. Soprano, M. L. Wyatt, and D. S. Goodman. 1989. An in situ-hybridization study of the localization of retinol-binding protein and transthyretin messenger RNAs during fetal development in the rat. *Differentiation.* 40:17–25.

24. Hart, M. N., D. D. Heistad, and M. J. Brody. 1980. Effect of chronic hypertension and sympathetic denervation on wall/lumen ratio of cerebral vessels. *Hypertension (Dallas).* 2:419–423.

25. Bevan, R. D., and H. Tsuru. 1981. Functional and structural changes in the rabbit ear artery after sympathetic denervation. *Circ. Res.* 49:478–485.

26. Lee, R. M. K. W., C. R. Triggle, D. W. T. Cheung, and M. D. Coughlin. 1987. Structural and functional consequence of neonatal sympathectomy on the blood vessels of spontaneously hypertensive rats. *Hypertension (Dallas).* 10:328–338.

27. Jackson, C. L., and S. M. Schwartz. 1992. Pharmacology of smooth muscle cell replication. *Hypertension (Dallas).* 20:713–736.

28. Hannan, R. D., and A. K. West. 1991. Adrenergic agents, but not triiodo-L-thyronine induce *c-fos* and *c-myc* expression in the rat heart. *Basic Res. Cardiol.* 86:154–164.

29. Iwaki, K., V. P. Sukhatme, H. E. Shubeita, and K. R. Chien. 1990. Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression on neonatal rat myocardial cells. fos/jun expression is associated with sarcomere assembly; *Egr*-1 induction is primarily an alpha 1-mediated response. *J. Biol. Chem.* 265:13809–13817.

30. Kawahara, Y., M. Sunako, T. Tsuda, H. Fukuzaki, Y. Fukumoto, and Y. Takai. 1988. Angiotensin II induces expression of the *c-fos* gene through protein kinase C activation and calcium ion mobilization in cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 150:52–59.

31. Naftilan, A. J., R. E. Pratt, and V. J. Dzau. 1989. Induction of platelet-derived growth factor A-chain and c-*myc* gene expressions by angiotensin II in cultured rat vascular smooth muscle cells. *J. Clin. Invest.* 83:1419–1424.

32. Taubman, M. B., B. C. Berk, S. Izumo, T. Tsuda, R. W. Alexander, and B. Nadal-Ginard. 1989. Angiotensin II induces *c-fos* mRNA in aortic smooth muscle. Role of Ca^{2+} mobilization and protein kinase C activation. *J. Biol. Chem.* 264:526–530.

33. Simonson, M. S., S. Wann, P. Mené, G. R. Dubyak, M. Kester, Y. Nakazato, J. R. Sedor, and M. J. Dunn. 1989. Endothelin stimulates phospholipase C, Na^+/H^+ exchange, c-*fos* expression, and mitogenesis in rat mesangial cells. *J. Clin. Invest.* 83:708–712.

34. Bylund, D. B. 1992. Subtypes of α_1- and α_2-adrenergic receptors. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:832–839.

35. Lomasney, J. W., L. F. Allen, D. Capel, and R. J. Lefkowitz. 1993. Distinct transmembrane signalling pathways coupled to different cloned subtypes of the α_1-adrenergic receptor. *Clin. Res.* 41:264*a*. (Abstr.)

36. Oriowo, M. A., and R. R. Ruffolo, Jr. 1992. Heterogeneity of postjunctional α_1-adrenoceptors in mammalian aortae: subclassification based on chlorethylclonidine, WB4101 and nifedipine. *J. Vasc. Res.* 29:33–40.

37. Ruffolo, R. R., Jr., and M. A. Oriowo. 1990. Evidence that different signal transduction mechanisms may be activated by a single α_1-adrenoceptor population in rat aorta. *Eur. J. Pharmacol.* 183:1601–1602.

38. Han, C., P. W. Abel, and K. P. Minneman. 1987. α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature (Lond.).* 329:333–335.

39. Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 266:15771–15781.

40. Godfraind, T. 1983. Actions of nifedipine on calcium fluxes and contraction in isolated rat arteries. *J. Pharmacol. Exp. Ther.* 224:443–450.

41. Scarborough, N. L., and G. O. Carrier. 1984. Nifedipine and alpha adrenoceptors in rat aorta. I. role of extracellular calcium in alpha-1 and alpha-2 adrenoceptor-mediated contraction. *J. Pharmacol. Exp. Ther.* 231:597–602.

42. Nishimura, K., M. Ota, and K. Ito. 1991. Existence of two components in the tonic contraction of rat aorta mediated by α_1-adrenoceptor activation. *Br. J. Pharmacol.* 102:215–221.

43. Krieger, J. E., and V. J. Dzau. 1991. Molecular biology of hypertension. *Hypertension (Dallas).* 18(Suppl.):I-3–I-17.